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# ABA Signaling Is Necessary but Not Sufficient for *RD29B* Transcriptional Memory during Successive Dehydration Stresses in *Arabidopsis thaliana*

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## **ABA Signaling Is Necessary but Not Sufficient for *RD29B* Transcriptional Memory during Successive Dehydration Stresses in *Arabidopsis thaliana***

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### **Abstract**

Plants subjected to a *prior* dehydration stress were seen to have altered transcriptional responses during a subsequent dehydration stress for up to 5 days after the initial stress. The abscisic acid (ABA) inducible *RD29B* gene of *Arabidopsis thaliana* was strongly induced after the first stress and displayed transcriptional memory with transcript levels nine-fold higher during the second dehydration stress. These increased transcript levels were due to an increased rate of transcription and are associated with an altered chromatin template during the recovery interval between the dehydration stresses. Here we use a combination of promoter deletion/substitutions, mutants in the *trans*-acting transcription factors and their upstream protein kinases, and treatments with exogenous ABA or dehydration stress to advance our understanding of the features required for transcriptional

memory of *RD29B*. ABA Response Elements (ABREs) are sufficient to confer transcriptional memory on a minimal promoter, although there is a context effect from flanking sequences. Different mutations in *Snf1 Related Protein Kinase 2* (*SnRK2*) genes positively and negatively affected the response, suggesting that this effect is important for transcriptional memory. Although exogenous ABA treatments could prime transcriptional memory, a second ABA treatment was not sufficient to activate transcriptional memory. Therefore, we concluded that transcriptional memory requires ABA and an ABA-independent factor that is induced or activated by a subsequent dehydration stress and directly or indirectly results in a more active *RD29B* chromatin template. These results advance our knowledge of the *cis*- and *trans*-acting factors that are required for transcriptional memory of *RD29B*.

**Keywords:** *Arabidopsis thaliana*, dehydration stress, transcriptional memory, abscisic acid, *RD29B*, ABRE, *SnRK2*

## Introduction

Several examples exist in which an initial stress or stimulus causes plants to alter their response during a similar second stress or signal. Pre-treatment (priming) with hormones (jasmonic acid, abscisic acid, or salicylic acid) increased systemic immunity and/or induced stronger or altered responses from the genes involved upon subsequent treatments relative to non-primed plants (Baldwin and Schmelz, 1996; Goh et al., 2003; Ton et al., 2005, 2007; Conrath et al., 2006; Conrath, 2011; Jaskiewicz et al., 2011; Ali et al., 2013). These observations have led to the concept of “stress memory,” implying that under-repeated exposures plants have faster and/or stronger responses than their response to an initial stress (Bruce et al., 2007). The molecular mechanisms responsible for priming of the plants during the initial stress are not well understood but have been proposed to involve accumulation of cellular proteins, such as signaling proteins and transcription factors, or involve an epigenetic mechanism (Conrath et al., 2006; Bruce et al., 2007). Priming or stress memory can be associated with histone H3 lysine 4 tri-methylation (H3K4me3) during the primed memory interval (Conrath, 2011; Ding et al., 2012; Kim et al., 2012).

Drought-triggered dehydration stress is one of the most common environmental stresses endured by plants, and a plant’s transcriptional responses to this stress have been extensively characterized (Nakashima and Yamaguchi-Shinozaki, 2013). Abscisic acid (ABA) is a key mediator of dehydration-stress signaling and much of the ABA signaling pathway has been elucidated recently (Fujita et al., 2011, 2013; Nakashima and Yamaguchi-Shinozaki, 2013). ABA binds to the ABA Pyrabactin Resistance/PYR1-like protein/Regulator component of the ABA receptor (PYR/PYL/RCAR) family receptor proteins (Cutler et al., 2010). The ABA-bound PYR/PYL/RCAR receptor complex binds to and inhibits the activity of the clade A PP2C phosphatases, which otherwise negatively regulate ABA signaling (Hirayama and Shinozaki, 2007). Upon binding to the ABA/receptor complex, PP2C phosphatases, including ABI1, ABI2, and HAB1, are no longer able to dephosphorylate *SnRK2* kinases, a gene family of serine/threonine protein kinases that are related to yeast SNF1 that mediate ABA-dependent and ABA-independent responses (Kulik et al., 2011). The active phosphorylated *SnRK2*s propagate ABA signaling (Klingler et al., 2010; Yunta et al., 2011; Xie et al., 2012) by phosphorylating many downstream target proteins, including bZIP transcription factors (TFs) belonging to the ABRE-binding factor (AREB/ABF)

family that regulate ABA-inducible genes (Fujita et al., 2005, 2009). These ABF TFs have been shown to be important for inducing ABA-mediated gene expression through the cis-acting ABA Response Elements (ABRE; ACGTGG/TC) (Yamaguchi-Shinozaki and Shinozaki, 1994; Uno et al., 2000; Fujita et al., 2013).

The ABA-inducible RD29A and RD29B genes encode dehydrin proteins that are evolutionarily related and adjacent to each other on chromosome 5. The ABA-inducible RD29A and RD29B promoters require ABRE consensus sequences for ABA inducibility (Yamaguchi-Shinozaki and Shinozaki, 1993; Uno et al., 2000). The RD29A promoter harbors both ABRE and dehydration-responsive elements (Narusaka et al., 2003). In contrast, the RD29B promoter has two adjacent ABRE elements that are more strongly dependent upon the presence of the ABF TF family members AREB1/ABF2, ABF3, and AREB2/ABF4 (hereafter ABF2, ABF3, and ABF4) for robust promoter activity (Uno et al., 2000; Fujita et al., 2005).

Recently, we demonstrated that RD29A and RD29B transcript levels in Arabidopsis plants subjected to several cycles of dehydration/water recovery treatments displayed different behaviors in their initial and subsequent responses (Ding et al., 2012). Both genes were induced during a first dehydration stress. After a watered recovery interval and during a second dehydration stress, RD29A was induced to a level similar to the first stress, i.e., the prior stress did not affect RD29A's behavior in a later stress. In contrast, RD29B was induced to levels nine-fold higher in the second stress, relative to its levels in the first dehydration (Ding et al., 2012). These changes in transcript levels were due to differences in the rates of transcription of RD29B during the first and subsequent stress. Furthermore, RD29B, but not RD29A, had H3K4me3 and paused RNA Polymerase II chromatin signatures that persisted during the watered recovery period. These features were associated with the subsequent increased transcriptional response at RD29B, indicative of a form of transcriptional memory (Ding et al., 2012). An Arabidopsis mutant for *abf2*, *abf3*, and *abf4* TFs (Yoshida et al., 2010) had greatly diminished RD29B transcript levels but still displayed transcriptional memory, suggesting the possible involvement of additional factors operating on the RD29B promoter during the transcriptional memory response in the subsequent stress (Ding et al., 2012).

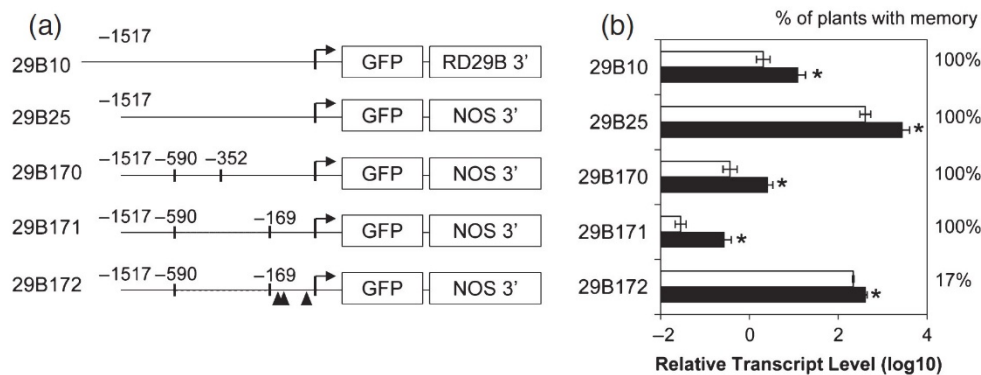
In this study, we used deletion and substitution mutants in the RD29B promoter to define the *cis*-acting transcriptional memory response elements. We also used exogenous ABA treatments and mutants in the ABA signaling pathway to better define the contributions of the ABA pathway to the transcriptional memory pathway of the RD29B gene. Our results elucidate the requirements for transcriptional memory at the ABRE-dependent RD29B promoter.

## Results

### *Analysis of the cis-acting elements of the RD29B promoter necessary for a transcriptional memory response*

The RD29B and RD29A genes are in the same orientation on chromosome 5, with the RD29B polyadenylation site 1.3 kb upstream of the RD29A transcription start site. We first determined that construct 29B10 that contained the RD29B promoter and 3' polyadenylation

region that expressed a GFP (*green fluorescent protein*) coding region displays transcriptional memory in transgenic plants (Fig. 1a,b). To determine if the 1.5 kb promoter region of *RD29B* is sufficient for the transcriptional memory response in transgenic plants, we compared the expression of the above construct, which contains a 1.4 kb 3' region of *RD29B*, to a similar construct 29B25 containing the Nopaline Synthase (NOS) 3' polyadenylation region (Fig. 1a). Both of these constructs displayed transcriptional memory (Fig. 1b), indicating competence for transcriptional memory is located in the 1.5 kb promoter region of *RD29B*. As the *RD29B* promoter fragments containing 5' positions to at least -494 appear fully functional (Yamaguchi-Shinozaki and Shinozaki, 1994), and because flanking sequences can affect promoter function, we then tested whether internal deletions between -590/-352 or -590/-169 displayed transcriptional memory (Fig. 1a). Analysis of GFP transcript levels after an initial (S1) and a third (S3) air-drying stress demonstrated that transgenic plants with these promoters in constructs 29B170 and 29B171 retained transcriptional memory (Fig. 1b). These results suggest that the *RD29B* promoter region between either -1.5 kb to -590 and/or -169 to +1 contains the *cis*-acting regions conferring competence for transcriptional memory.

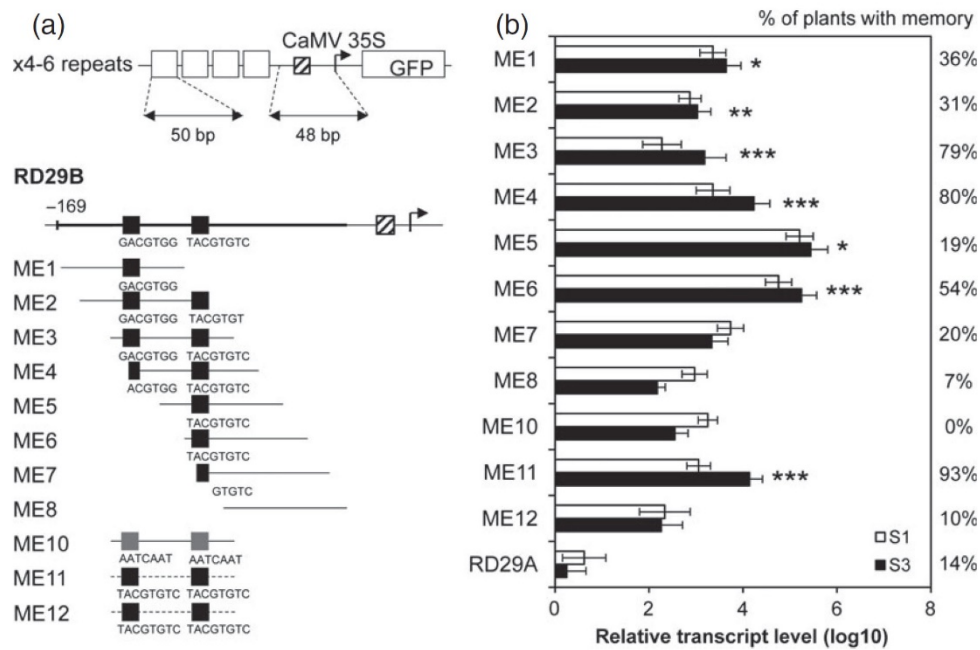


**Figure 1.** Analysis of *RD29B* regions required for transcriptional memory. (a) Schematic diagrams of the *RD29B* promoter from -1517 to the transcription start site (arrow at +1) and deletions (dotted lines) or base substitutions (black triangles) therein, the GFP transcribed region, and either the native *RD29B* or nopaline synthase (NOS) 3' polyadenylation regions. (b) *RD29B* transcript levels from four to six independent 3-week-old transgenic plants were analyzed by qRT-PCR after 2 h of an initial (S1; white bars) and a third (S3; black bars) air-drying stress. The percentage of independently transformed plants displaying transcriptional memory ( $S3/S1 \geq 3$ ) is shown on the right (see Table S1 for alternative calculations). The S1 and S3 values correspond to the mean of the  $\log_{10}$ -transformed values for four to six independent transgenic plants for each condition, with the standard error of the mean shown. An asterisk indicates the constructs where S3 is significantly different than S1 by a *t*-test on the  $\log_{10}$ -transformed values at  $P < 0.01$ .

A motif analysis of the promoters of the two memory genes, *RD29B* and *RAB18*, revealed that, in addition to ABREs, the two promoters shared homology at the 5-bp regions flanking either side of the upstream ABRE element (GACGTGGC) at -138/-131 as well as

with a sequence located at –60/–43 of *RD29B* (GTTCGGCCATATGTCATC). Therefore, as a rapid test of whether any of these regions are important to transcriptional memory, we constructed *RD29B172* (Fig. 1a), which in addition to a –590/–169 deletion also contains sequence substitutions at these three potential motifs. Transcriptional memory in *RD29B172* was weak (Fig. 1b), indicating that sequences within the –169/–43 *RD29B* promoter region were important for transcriptional memory. Note that the –169/–43 region contains two ABREs that are required for the ABA inducibility of *RD29B* (Uno et al., 2000), and that these are intact in *RD29B172*. This result indicates the flanking substitutions in *RD29B172* affect the strength of the memory response in the presence of intact ABREs. The weak transcriptional memory response of *RD29B172*, relative to the other constructs tested (Fig. 1 and Table S1), also indicates that the transcriptional memory responses are caused by *cis*-acting sequences and are not a general consequence of the transformation/integration process. This conclusion is further supported by the analysis of the non-memory *RD29A* promoter (presented below).

To more precisely localize the *cis*-acting elements in the –169/–43 region of *RD29B*, we multimerized different 50-bp sections of this region upstream of a minimal –47 Cauliflower Mosaic Virus (CaMV) 35S promoter expressing GFP (Fig. 2a). Note the only sequences from *RD29B* in this new set of constructs are the 50-bp multimerized sections from the –169/–43 region of *RD29B*, as the upstream –1.5 kb to –590 and –42/+1 promoter regions of *RD29B* are not present (Fig. 2a). Transgenic plants that containing these ME1–8 and ME10 constructs were analyzed for their GFP transcript levels during a first and third dehydration stress (Fig. 2a and Tables S2 and S3). Endogenous *RD29B* transcripts served as an internal control to verify that the transgenic plants were displaying dehydration responses during these analyses (Table S3).



**Figure 2.** Ability of 50 bp regions of the *RD29B* promoter to confer transcriptional memory on a minimal CaMV 35S promoter. (a) Schematic of the structure for testing 50-bp regions of the *RD29B* promoter, as a tandem array of four to six repeats upstream of a -47 CaMV 35S minimal promoter expressing GFP. The location of the -169/+1 region *RD29B*, of the two ABREs (black boxes), the TATA box at -35 (striped box) and transcription start site (arrow) of the *RD29B* promoter are also shown. Below this are the 50 bp of wild-type *RD29B* sequences contained in constructs ME1–ME8. Constructs ME10–ME12 correspond to the same sequence as ME3 except ME10 contains substitutions in the ABRE regions (gray boxes) and constructs ME11 and ME12 contain two ABREs within randomly generated flanking sequences (dotted lines). (b) GFP RNA levels were measured from 3-week-old transgenic plants by qRT-PCR after 2 h of air-drying for a first dehydration stress (S1; white bars) or a third dehydration (S3; black bars). The GFP transcript levels were measured in 10–15 independent transgenic plants for each *RD29B* construct as well as for a control *RD29A* construct. The values correspond to the mean of the log<sub>10</sub>-transformed expression values, with the standard error of the mean shown. The percentage of independently transformed plants displaying transcriptional memory (S3/S1 ≥ 3) is shown on the right (see Table S2 for alternative calculations). One, two, or three asterisks indicate constructs for which the S3/S1 ratio is significantly different than the *RD29A* control by a *t*-test on the log<sub>10</sub>-transformed values at  $P \leq 0.1$ ,  $P < 0.05$ , and  $P < 0.01$ , respectively.

A promoter control with a similar 50-bp multimer structure expected to lack transcriptional memory was derived from the promoter of *RD29A*, which does not display transcriptional memory (Ding et al., 2012). The 50-bp sequence contains the *as1* (GACGTC) and ABRE element from the *RD29A* promoter as a 4× tandem repeat upstream of the minimal CaMV 35S promoter. This multimerized region corresponds to the region used in construct D4 (Narusaka et al., 2003). Transgenic plants with this *RD29A* construct generally had

lower S3 than S1 values and did not display transcriptional memory (Fig. 2b). This lack of memory response indicates the transgene reflects the behavior of its endogenous counterpart and that the random sites of chromosomal insertion are not conferring transcriptional memory.

The transcriptional memory responses of the *RD29B* tandem repeat constructs ME1–8 and ME10 in transgenic plants fell into two classes: those lacking transcriptional memory, and those with various strengths of transcriptional memory (Fig. 2). Our summary value for transcriptional memory is the percentage of independent transgenic plants displaying transcriptional memory, defined herein as a S3/S1 ratio of three or greater. For comparison, the non-memory *RD29A* construct has a value of 14%, as two of the 14 independent transgenic plants had S3/S1 ratios of three or greater. Constructs ME8 and ME10 did not display transcriptional memory, while ME7 had little or no memory as it was just above the *RD29A* value at 20% (Fig. 2). ME7 and ME8 lack intact ABRE sequences, and ME10 lacks ABREs due to complete sequence substitutions within its two ABREs (Fig. 2a). In contrast, constructs ME1–ME6 containing one or two multimerized ABREs display higher levels of transcriptional memory; multimers of two ABREs generally provided stronger responses than multimers of single ABREs (Fig. 2b). Of note is the comparison of ME10 and ME3, which span the same region. ME10 has sequence substitutions at the ABREs and does not demonstrate transcriptional memory, while ME3, which contains two ABREs, shows strong transcriptional memory.

To more stringently test the ability of the two ABREs to confer transcriptional memory, the flanking sequences in the 50-bp regions of ME3 were randomized, such that only the two ABRE sequences were retained from the original *RD29B* sequence in this region (Fig. 2a). Two separate randomized sequences containing embedded ABREs were tested in transgenic plants. The first, ME11, displayed strong transcriptional memory, while the second, ME12, did not (Fig. 2b).

An alignment of the sequences of the construct 29B172 (Fig. 1) and selected ME constructs from Figure 2 is presented in Figure 3 to help visualize the sequence changes occurring in the vicinity of the two ABRE elements in this region of the *RD29B* promoter (Figure 3). 29B172 has changes flanking its upstream ABRE and has weak S3/S1 memory. ME2 has changes downstream of its downstream ABRE and also has weak S3/S1 memory. ME12 has randomized sequences flanking its two ABREs and does not have S3/S1 memory. These data lead to the conclusion that sequence changes flanking the intact ABREs can attenuate transcriptional memory responses.



	10	20	30	40	50	% of plants with memory
29B172	ACTGcgcacGACGTGGCAcactGAAACGGA-CGCATCGTACGTGTCAGAAAT					17%
ME2	ACTGGCTGAGACGTGGCAGGACGAAACGGA-CGCATCGTACGTGTCgGAcc					31%
ME3	ACTGGCTGAGACGTGGCAGGACGAAACGGA-CGCATCGTACGTGTCAGAAAT					79%
ME4	ACgcGgaccGACGTGGCAGGACGAAACGGA-CGCATCGTACGTGTCAGAAAT					80%
ME5	AagtaaaGAGACacGGaccGACGAAACGGA-CGCATCGTACGTGTCAGAAAT					19%
ME6	AagtaaaGAGACagaagccagaGcggaccg-CGCATCGTACGTGTCAGAAAT					54%
ME11	ggTaatacagtACGTGtCtGaAgtAgggtaAgtatgctaTACGTGTCgagggc					93%
ME12	gagaGtTcgtACGTGtCAGttAtctgggttcgcgCgaCGTACGTGTCtGcga					14%

**Figure 3.** Sequence alignment of constructs spanning one or two ABREs. The construct names and the percentage of plants displaying transcriptional memory are from Figures 1 and 2. The two ABREs (red box), native (black capitalized) or mutated (blue lower case) flanking nucleotides are aligned and shown. The spacing between the ABRE elements is one nucleotide more in ME11 and ME12, as indicated by the hyphen (–) in the sequences aligned above these.

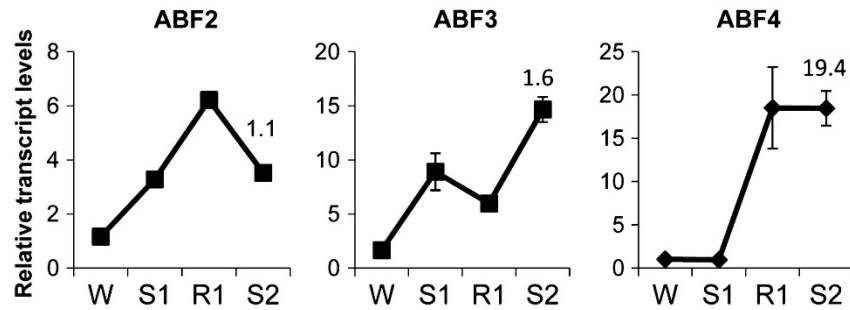
ME5 and ME6 contain multimers of a sequence containing only a single downstream ABRE. Therefore these constructs lack the entire region containing the upstream ABRE and yet display some S3/S1 memory, particularly ME6. ME3 and ME4 contain both ABREs, with ME4 having substitutions just upstream of its upstream ABRE, and both have strong S3/S1 memory. ME11 has a complete substitution of flanking sequences and also displays strong S3/S1 memory. Together these constructs substitute for all of the sequences flanking the two ABREs and do not identify a distinct non-ABRE region critical for S3/S1 memory.

#### *The involvement of ABF transcriptional factors*

ABF2, ABF3, and ABF4 bind the ABREs at the *RD29B* promoter (Uno et al., 2000; Fujita et al., 2005). In our earlier study (Ding et al., 2012), also confirmed here (Figure S1), we found that *RD29B* transcript levels induced in S1 in the triple mutant *abf2/abf3/abf4* background were <1% of wild-type expression; nonetheless, transcript levels in S2 were higher than in S1, consistent with a transcriptional memory response (note one cycle of prior to dehydration stress was found to be adequate for memory responses and was less stressful to the plants than two cycles; therefore S2/S1 ratios were used hereafter). This result was previously interpreted to support the role of a non-ABF TF at the *RD29B* promoter (Ding et al., 2012). However, in light of the results above indicating that ABREs are necessary and can be sufficient for transcriptional memory, the role of ABF2/3/4 during S1 and S2 was further investigated. Although, *ABF2*, *ABF3*, and *ABF4* expression are known to be induced by abiotic stress (Fujita et al., 2005), the transcript and protein levels of these genes during multiple dehydration stresses is unknown. Therefore, we analyzed the transcript levels of *ABF2*, *ABF3*, and *ABF4* during successive dehydration stresses to test the hypothesis that increased levels of these TFs mediate increased *RD29B* expression levels during repeated stresses.

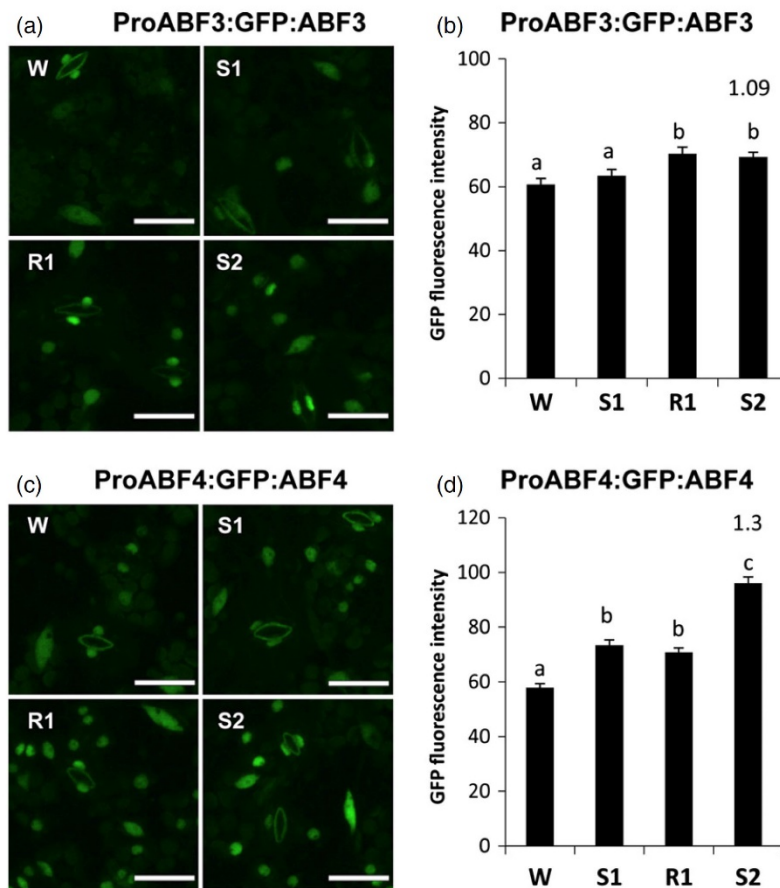
At the transcript level, *ABF2* and *ABF3* are induced, but *ABF4* is not, during the first dehydration stress in our air-drying stress system (Figure 4). After the first induction, although

ABF2 transcript levels rose slightly during the recovery period, ABF2 transcripts were present at similar levels in the S1 and S2 responses. In contrast, ABF3 transcript levels were 1.6-fold higher in a subsequent S2 stress and ABF4 transcript levels were 19-fold higher in S2 (Figure 4). Because the ABF3 and ABF4 genes displayed transcriptional memory behavior, we analyzed the pattern of their protein levels during the repeated stresses.



**Figure 4.** Analysis of ABF2, ABF3, and ABF4 transcript levels in response to successive dehydration stresses. Transcript levels of ABF2, ABF3 and ABF4 were measured by qRT-PCR on 3-week-old seedlings that were either watered (W), air-dried for 2 h (S1), recovered by watering for 22 h (R1), or air-dried for 2 h after recovery (S2). Points correspond to the values of a representative experiment, mean  $\pm$  SEM of three technical replicates. The experiment was repeated at least three times. The values above the S2 data point correspond to the S2/S1 ratio.

The *ABF3* and *ABF4* coding regions were fused to *GFP* and expressed from their respective native promoters, using new constructs and transgenic plants recapitulating those described earlier (Yoshida et al., 2010). The transcripts of these chimeric genes demonstrated transcriptional memory behavior in transgenic plants (Figure S2). The fusion protein levels were too low to be detected by immunoblot analysis using GFP-antibody (data not shown). Therefore, ABF-GFP-fusion protein levels and the subcellular localization of the GFP-fusion proteins were visualized by confocal microscopy during a first and second dehydration stress (Figure 5). Both ABF3-GFP and ABF4-GFP proteins were targeted to the nucleus of the epidermal and guard cells (Figure 5a,c) as previously reported (Yoshida et al., 2010). ABF3-GFP protein levels were only 1.1-fold higher during the second stress (Figure 5a,b). Surprisingly, the ABF4-GFP protein was only 1.3-fold higher during the subsequent stress period (Figure 5c,d), despite the large change in *ABF4* transcript levels. These results indicated that ABF4 protein levels are post-transcriptionally regulated as their protein levels do not correlate with their transcript levels. Therefore, we conclude the 1.1- and 1.3-fold increases in ABF3 and ABF4 proteins, respectively, contribute to, but are probably not solely responsible for, the nine-fold increase in *RD29B* transcript levels in S2 relative to S1. This result suggests that another factor is probably involved in the transcriptional memory response.



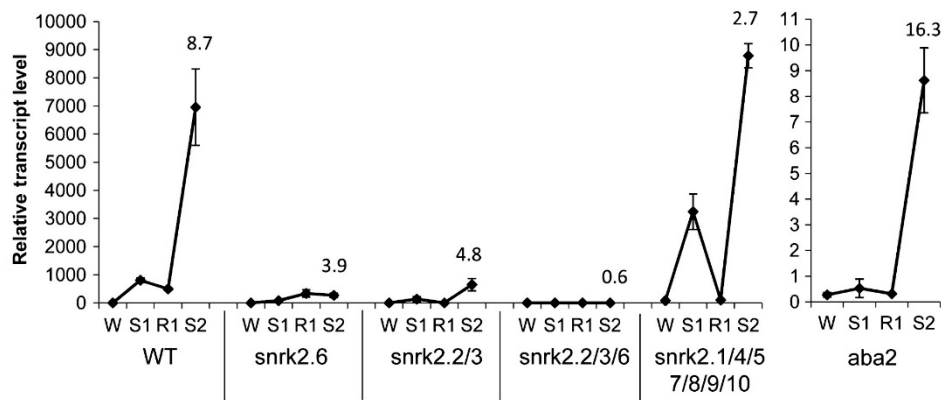
**Figure 5.** Analysis of GFP-ABF3 and GFP-ABF4 fusion protein levels. Transgenic 3-week-old seedlings containing constructs with the ABF3 or ABF4 promoters expressing GFP fused to the N-terminus of the ABF3 or ABF4 cDNAs, respectively, were analyzed for GFP protein levels in plants that were either watered (W), air-dried for 2 h (S1), recovered by watering for 22 h (R1), or air-dried for 2 h after recovery (S2). (a, c) Confocal images of GFP fluorescence in leaf epidermal tissues of ProABF3:GFP-ABF3 line 30 and ProABF4:GFP-ABF4 line 23, respectively. Bars = 5  $\mu$ m for (a) and (c). (b, d) The mean ( $\pm$  SEM) GFP fluorescence intensities were measured from nuclei in the confocal images with ImageJ software. GFP intensities in 120–280 nuclei were measured in three or four plants of each line, for each of the four conditions shown. Different letters above the error bars indicate significant differences by *t*-test ( $P < 0.05$  for ABF3 and  $P < 0.01$  for ABF4). The values above the S2 data point correspond to the S2/S1 ratio.

#### *The ABA signaling pathway in the RD29B memory response*

Next, we examined whether an ABA-independent factor could operate on the native *RD29B* promoter in the presence of ABF2, ABF3, and ABF4, but in the absence of ABA signaling. To accomplish this examination we analyzed the transcriptional memory response in knockout mutants in the ABA-dependent SnRK2 pathway. Recent studies have revealed that the three subclass II SnRK2 kinase proteins, SRK2D/SnRK2.2, SRK2E/SnRK2.6,

and SRK2I/SnRK2.3 play a role in ABA signaling (Fujii and Zhu, 2009b), whereas the seven other SnRK2s are involved in ABA-independent pathways for osmotic stress response (Fujii et al., 2011). Thereby, the *snrk2.6* single, *snrk2.2/3* double, *snrk2.2/3/6* triple, and *snrk2.1/4/5/7/8/9/10* septuple mutants were examined for their effects on *RD29B* expression.

Interestingly, the transcript levels of *RD29B* during the initial and subsequent dehydration stresses were elevated in the *snrk2.1/4/5/7/8/9/10* septuple mutant, suggesting that these ABA-independent SnRK2 proteins are not required to positively regulate, and in fact negatively regulate, dehydration stress signaling (Figure 6). In contrast, the *RD29B* transcript induction in response to the first dehydration stress was extremely low or absent in the single *snrk2.6*, double *snrk2.2/3*, and the triple *snrk2.2/3/6* mutants (Figure 6). In the subsequent transcriptional memory response assay, *RD29B* had a weak transcriptional memory response in the single *snrk2.6* and double *snrk2.2/3* mutants (Figure 6). However, *RD29B* expression was completely abolished during both the first and the second dehydration stresses in *snrk2.2/3/6* triple mutants. This result indicates that SnRK2.2, SnRK2.3, and SnRK2.6 are essential for transcriptional memory at *RD29B*. Further, the *RD29B* promoter cannot respond in S1 or S2 in the absence of SnRK2 function. This finding supports the conclusion that there is not an ABA-independent *cis*-acting memory site in the *RD29B* promoter.



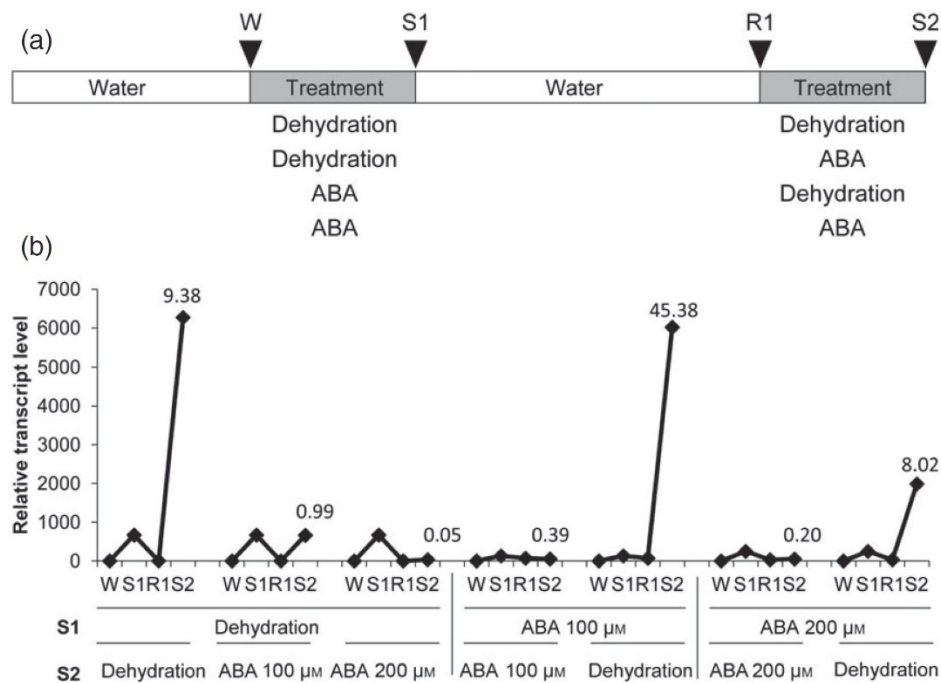
**Figure 6.** Positive and negative effects of SnRK2s and ABA on transcriptional memory responses of *RD29B*. Three-week-old mutant and wild-type C010 (WT) plants were analyzed for *RD29B* transcript levels by qRT-PCR and were either watered (W), air-dried for 90 min (S1), recovered by watering for 22 h (R1), or air-dried for 90 min after recovery (S2). The relative transcript levels are indicated on the *y*-axis, the genotypes and treatments are indicated along the *x*-axis, and the S2/S1 ratio is indicated above the S2 value. The values correspond to a representative experiment, mean  $\pm$  SEM of three technical replicates. The experiment was repeated at least three times. Note the expanded *y*-axis scale for the *aba2* data to visualize the low transcripts levels in this genotype.

Furthermore, a mutation in *aba2* (Nambara et al., 1998), which is involved in ABA biosynthesis, reduced *RD29B* transcript levels to <1% of wild-type levels but still displayed an attenuated memory response in S2 (Figure 6). These results demonstrate the necessity

of the ABA signaling pathway in both the initial response (in S1) and in the memory response (in S2), but it remained unclear whether ABA was sufficient for the memory response. This question was addressed below.

***ABA versus dehydration-stress treatments in the memory response of the RD29B***

If only ABA signaling is involved in the transcriptional memory response, then exogenous ABA should be sufficient to prime memory in “S1” and to activate the memory response in “S2.” This hypothesis was analyzed by substituting exogenous ABA treatments for the dehydration-stress treatments. We examined all four combinations of ABA and dehydration-stress treatments (Figure 7a). The effects of these treatments on *RD29B* transcript levels were then measured (Figure 7b). Exogenous ABA treatments induced *RD29B*, but a sequential ABA treatment with the second ABA treatment after a recovery interval, could not induce transcriptional memory (Figure 7b). Surprisingly, a first ABA treatment could prime transcriptional memory as long as the second treatment was dehydration stress. Therefore, ABA treatment is sufficient for priming transcriptional memory but not for activating a transcriptional memory response (Figure 7b). In support of this conclusion, an initial dehydration stress followed by exogenous ABA as the second treatment was also not sufficient to cause a transcriptional memory response (Figure 7b). Apparently, there is a dehydration-dependent, ABA-independent component required for active transcriptional memory that is not induced or activated by ABA alone during a second ABA treatment.



**Figure 7.** *RD29B* transcriptional memory in response to different combinations of ABA and/or dehydration-stress treatments. (a) Schematic view of how the different treatments with ABA or air-drying dehydration stresses were applied. Leaves were harvested (inverted triangles) at the watered condition (W), after 2 h of air-drying stress or ABA treatment at 100 or 200  $\mu$ M (S1), after washing and 22 h of recovery in water (R1), and after 2 h of air-drying stress or ABA treatment at 100  $\mu$ M or 200  $\mu$ M (S2). (b) *RD29B* relative transcript levels are indicated on the y-axis, and the S1 and S2 designations at the left lower corner indicate which treatment occurred in S1 and which occurred in S2. The values correspond to a representative experiment, mean  $\pm$  standard error of the mean (SEM) of three technical replicates. The experiment was repeated at least three times. The values above the S2 data points are the S2/S1 ratio observed.

## Discussion

### *ABREs can confer transcriptional memory and are affected by flanking sequences*

Previous studies demonstrated that the two adjacent ABRE elements present in the *RD29B* promoter are required for ABA-inducible expression of *RD29B* (Uno et al., 2000; Nakashima et al., 2006). Our promoter deletion and substitution analysis indicates that the two adjacent ABREs are also necessary for, and can confer, transcriptional memory to a minimal promoter, depending on the flanking sequences. In our most extreme substitution construct ME11, the presence of two ABREs in a synthetic random repeat upstream of the minimal CaMV35S promoter was sufficient to confer robust transcriptional memory. Collectively, our deletion and substitutions removed all the flanking sequences adjacent to the two ABREs, but no specific sequence, other than the ABREs, emerged as being required

for transcriptional memory. However, there does appear to be a DNA context or flanking sequence effect. This observation is supported by results from several constructs: (i) ME12, a second construct with two ABREs embedded in random flanking sequences, did not display transcriptional memory; (ii) transcriptional memory was affected by deletion boundaries close to the ABRE consensus sequences as in the case of ME2 and ME4; and (iii) a multimer containing the ABRE from the non-memory RD29A promoter did not show transcriptional memory while constructs ME5 and ME6, which contain multimers of a single ABRE from RD29B, did display transcriptional memory.

Flanking sequence or context effects have been observed at other promoters such as those containing W-boxes recognized by WRKY TFs (Ciolkowski et al., 2008). However, we note that substitution of TF binding sites into random sequences upstream of minimal promoters is rarely performed experimentally, and therefore the predicted “success rate” of such experiments is not known. Therefore, the interpretation of a mixed result such as a negative result from ME12 and a positive result from ME11 (Figures 2 and 3) can either be that there are flanking sequence effects or possibly a more specific hidden motif in ME11. Further insight can be gained by comparing all the sequence substitutions (Figure 3), which collectively do not appear to support the requirement for a specific motif. We conclude that our results do not provide support for an independent *cis*-acting sequence that confers transcriptional memory or for any unique flanking sequence that is required for transcriptional memory. However, the flanking sequence effects can explain why some ABRE-containing promoters display transcriptional memory and why some do not, without requiring a specific flanking motif.

#### ***Residual memory in mutants and the role of SnRK2s***

Plants mutant in *abf2/3/4* have an attenuated initial dehydration stress response (Yoshida et al., 2010) but maintain a transcriptional memory response from RD29B that is lower in absolute levels but is still strong in terms of the relative ratio of the first and a subsequent stress (Ding et al., 2012). The residual weak activity could be due to the limited activity of another member of the ABF family, such as ABF1, or possibly binding of a non-ABF protein to the ABREs. In support of this observation, the AtbZIP1 transcription factor was found to be involved in the regulation of ABA-responsive genes through its binding to ABRE *cis*-elements (Sun et al., 2011). Similarly, the *aba2* mutant, which has very low ABA levels (Nambara et al., 1998), retains some transcriptional memory (Figure 6). Presumably these low ABA levels are too low to trigger RD29B expression in S1, but the transcriptional memory response is capable of responding to these low levels in S2, although transcript levels of this response are only 1% of wild-type (WT). Taken together with the promoter mutation analysis above, these results suggest that the residual transcriptional memory responses are occurring via the ABREs.

Our results with the SnRK2 mutants indicate the residual transcriptional memory in the *abf2/3/4* triple mutant is SnRK2.2, SnRK2.3, and SnRK2.6 dependent as the triple *snrk2.2/3/6* mutant completely eliminated the transcriptional response to the initial stress and the subsequent stress-induced transcriptional memory. Taken together, these results suggest SnRK2.2, SnRK2.3, and SnRK2.6 proteins are essential not only for an initial RD29B dehydration-stress response (Fujii and Zhu, 2009b; Fujita et al., 2009) but also for the subsequent

transcriptional memory response at *RD29B*. The simplest interpretation of the SnRK2.2, SnRK2.3, and SnRK2.6 dependency is a lack of activation of ABFs as their activation depends on these kinases (Fujii et al., 2009a; Fujita et al., 2013). Importantly, these results demonstrate that no significant transcriptional memory activity occurs on the intact endogenous *RD29B* promoter in the absence of SnRK2.2, SnRK2.3, and SnRK2.6 signaling, i.e., there is not an independent *cis*-acting site and corresponding *trans*-acting factor that have any measurable effect on dehydration-induced transcription in the absence of SnRK2.2, SnRK2.3, and SnRK2.6 activation.

Mutations in the ABA-independent signaling SnRK2 pathway (*snrk2.1/4/5/7/8/9/10*) resulted in higher S1 and S2 responses, indicating a repressive role in transcriptional memory for members of this SnRK2 subgroup. A similar negative role has been noted for rice SAPK6 (a homolog of SnRK2.1/4/5/9/10) as its overexpression in tobacco plants was associated with ABA insensitivity (Chae et al., 2007). Additional support for a negative role is the observation that *snrk2.1/4/5/9/10* Arabidopsis plants have an increased sensitivity to ABA (Fujii et al., 2011). The absolute dependence of *RD29B* expression on SnRK2s in S1 and S2, as well as the large increase in S1 and S2 stimulated *RD29B* transcript levels in the *snrk2.1/4/5/7/8/9/10* mutant (Figure 6), suggests that SnRK2s are a regulatory node for transcriptional memory processes for *RD29B*.

#### ***ABF2, ABF3, and ABF4 protein levels weakly correlate with transcriptional memory***

Transcriptional induction of some yeast and human genes occurs with faster kinetics if the gene has been previously expressed, providing another example of transcriptional memory (Laine et al., 2009; Guan et al., 2012; Light et al., 2013). The SWI/SNF complex, histone variant H2A.Z, and components of the nuclear pore have been considered as factors in this memory behavior (Laine et al., 2009; Tan-Wong et al., 2009; Kundu and Peterson, 2010; Light et al., 2010). Other studies, however, have suggested that the memory behavior of the *GAL* genes system does not have a chromatin basis but, rather, memory of the previous transcription state is controlled primarily by the persistence of the cytoplasmic Ga13p and Ga11p signaling factors synthesized during the first stress (Zacharioudakis et al., 2007; Kundu and Peterson, 2010).

Accordingly, we considered increased ABF2, ABF3, and ABF4 protein levels as a potential mechanism for the increased transcription rate that occurs during transcriptional memory (Ding et al., 2012). ABF2, ABF3, and ABF4 protein or transcript levels have been shown to be expressed at low levels in unstressed plants and to be moderately induced by ABA and osmotic stress such as dehydration and high salinity (Uno et al., 2000; Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005). Overexpression of a wild-type ABF2 protein had little effect on *RD29B* expression, but overexpression of a constitutively active form of ABF2 induced the expression of target genes including *RD29B* without dehydration stress (Fujita et al., 2005). ABF TF proteins require activation by phosphorylation by SnRK2s for activity (Uno et al., 2000; Fujii et al., 2009a; Fujita et al., 2009). Here, we observed that although ABF2 does not change its transcript levels, ABF3 and ABF4 did increase their transcript levels but only slightly increased their protein levels between S1 and S2 dehydration stresses. Therefore, our results do not support large changes in ABF2, ABF3, and ABF4 protein levels as a mechanism for increased transcription during transcriptional memory



response. This result leaves open the possibility that the slightly increased protein levels, together with increased activity levels of these proteins, by phosphorylation or by other unknown modifications and/or protein interactions, contribute to transcriptional memory.

#### ***ABA is necessary but not sufficient for transcriptional memory***

Our finding that the SnRK2/ABF/ABRE pathway is essential for *RD29B* transcriptional memory is consistent with prior studies indicating this pathway is critical for the initial induction of *RD29B* (Nakashima and Yamaguchi-Shinozaki, 2013). We were unable to identify a separable *cis*-acting DNA region required for transcriptional memory. That is, there does not appear to be a requirement for any specific *cis*-acting DNA sequences as collectively these were all substituted while maintaining the ABRE-dependent transcriptional memory response. Further, if there is a separate *cis*-acting memory component, it does not have a measurable effect when SnRK2.2, SnRK2.3, and SnRK2.6 signaling is defective. These observations raised the question of whether exogenous ABA signaling is sufficient for transcriptional memory.

Exogenous ABA was necessary and sufficient for memory priming as a subsequent dehydration stress displayed transcriptional memory. However, ABA primed plants did not display transcriptional memory when treated with ABA in a subsequent treatment. Additionally, dehydration stress-treated plants did not display transcriptional memory when subsequently treated with ABA. We conclude there is an unknown factor that is activated or induced by an initial priming treatment, by either ABA or dehydration stress. This priming factor requires additional activation or interaction with an ABA-independent component of the subsequent dehydration stress response. Exogenous ABA is not sufficient to activate transcriptional memory in this subsequent treatment period.

These observations support a model wherein either ABA or an initial dehydration stress primes a memory factor. After a recovery interval, a second dehydration stress can activate this priming factor to cause transcriptional memory. The ABA/SnRK2/ABF/ABRE components of the ABA signaling pathway are necessary but not sufficient for the transcriptional memory response as sequential ABA treatments do not trigger transcriptional memory. The *RD29B* rate of transcription is higher during dehydration stress of a previously primed plant (Ding et al., 2012), indicating *cis*-acting changes in the chromatin template are facilitating increased rates of transcription. Our hypothesis is that the ABREs recruit the transcriptional memory components to the chromatin template via ABF2, ABF3, and ABF4. We demonstrate that any potential memory factor is unable to activate the *RD29B* promoter when SnRK2.2, SnRK2.3, and SnRK2.6 are lacking, presumably because ABF2, ABF3, and ABF4 are not activated.

Further, we hypothesize that the unknown memory component directly or indirectly results in ABFs modifications or protein interactions that facilitate a higher transcription rate via ABF2, ABF3, and ABF4 interaction with the ABREs. This outcome could be accomplished via changes in the activities of the SnRK2s. This model is supported by the increased *RD29B* transcript levels in S1 and S2 in the *snrk2.1/4/5/7/8/9/10* septuple mutant, suggesting the SnRK2 regulatory hub is capable of causing super-induced transcript levels. Alternatively, other factors such as phosphatases that dephosphorylate SnRK2.2, SnRK2.3, and SnRK2.6 or the ABF proteins, or a protein that binds ABF2, ABF3, and ABF4 before or

after they bind the ABREs are also possible mechanisms. Importantly, this process has an ABA-independent component as ABA signaling alone is not sufficient for this transcriptional memory. Although the above models can potentially explain the faster transcription rates observed during the transcriptionally active portions of the memory response (Ding et al., 2012), the presence of elevated H3K4me3 levels and paused RNA Polymerase II on the chromatin during the less transcriptionally active recovery phase (Ding et al., 2012) is harder to explain and suggests that the primed ABF/ABREs are able to recruit other proteins that participate in this potentiation of the chromatin template.

## Experimental Procedures

### *Plant growth and treatments*

Wild-type (Columbia-0), *abf2/3/4* (Yoshida et al., 2010), *snrk2.6*, *snrk2.2/3*, *snrk2.2/3/6*, *snrk2.1/4/5/7/8/9/10* (Fujii et al., 2011) and *aba2* (CS6147 allele) mutant Arabidopsis plants were grown in potting soil in growth rooms at 22°C with a 12-h light photoperiod and light intensity of 180  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ . The day before any treatment, the plants were removed from soil, any remaining soil from their roots was washed and their roots were placed in water during the night. The dehydration stress was applied as described (Ding et al., 2011). Briefly, 3-week-old water plants (W) were gently blotted onto filter paper to remove water and subjected to an initial air-drying stress for 2 h (S1). The plants were placed in water for 22 h, corresponding to the recovery period (R1). For a subsequent stress treatment, R1 plants were blotted onto filter paper to remove water and air-dried for 2 h (S2) followed by a recovery (R2). The same procedures were repeated for S3. One leaf from each of 8–10 plants was harvested at the different stress or recovery stages and stored in liquid nitrogen.

### *RD29B constructs 29B10, 29B25, 29B170, 29B171, and 29B172*

These constructs were made by standard recombinant DNA methods. The constructs are described in Methods S1. The Arabidopsis plants were transformed by floral dip method (Bent, 2006) and selected by red fluorescence on the seeds conferred by the binary vector (Stuitje et al., 2003). Four to six independently transformed plants were analyzed for each construct. The statistical analyses were performed by Student's *t*-test on the  $\log_{10}$  transformed values, to compare the expression between the initial (S1) and the third (S3) stress (Table S1).

### *Tandem repeat constructs*

To create a tandem repeat of the *RD29B* promoter regions, short oligonucleotides with flanking RsrII sites (Table S4) were cloned into an RsrII site upstream of the minimal –47 promoter of the CaMV 35S promoter, with external flanking *Bam*HI and *Xho*I sites. Multimers of the *RD29B* promoter region were identified by polymerase chain reaction (PCR) and sequenced, and the resulting *Bam*HI to *Xho*I promoter fragment was subcloned upstream of a GFP/NOS 3' cassette in a T-DNA binary vector. These binary plasmids were introduced into *Agrobacterium tumefaciens* by electroporation by using a GenePulser Xcell (Bio-Rad, Hercules, California, USA, <http://www.bio-rad.com/>). Transformation of Arabidopsis plants was performed by a floral dip method (Bent, 2006) and selected by red

fluorescence on the seeds conferred by the binary vector (Stuitje et al., 2003). Transgenic plants were analyzed for the expression of their endogenous RD29A gene to verify induction by dehydration stress and to have a S3/S1 ratio of at least 1. Of the independently transformed plants meeting these criteria, at least 13 independently transformed plants were analyzed for each construct, except for ME12 for which 10 plants were analyzed. The statistical analyses were performed by Student's *t*-test on the  $\log_{10}$  transformed values, to compare the expression between the initial (S1) and the third (S3) stress and the S3/S1 ratio of one construct against the RD29A construct (Tables S2 and S3).

#### **Reverse transcription and real-time PCR**

Total RNA isolation with TRIzol (Invitrogen, Grand Island, New York, USA, <http://www.lifetechnologies.com/us/en/home/brands/invitrogen.html>) was carried out as described (Virlovet et al., 2011). Subsequent DNase treatment and DNase inactivation steps to remove genomic DNA were performed. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen) with random primers (Invitrogen), and the amounts of individual genes transcript were measured with gene-specific primers: for GFP 5'-CGTCAACAGGATCGAGCTTA-3' and 5'-CTTGAAGTTGGCTTTGATGC-3', for RD29B (At5g52300) 5'-ACGAGCAAGACCCAGAAAGTT-3' and 5'-AGGAACAATCTCTCCGATG-3', for UBQ10 (AT4G05320) 5'-AGGATGGCAGAACTCTTGCT-3' and 5'-TCCCAGTCAACGTCTTAACG-3'. Real-time PCR analysis was performed with the cyclerIQ real-time PCR instrument (Bio-Rad) and SYBR Green mixture (Bio-Rad). The relative expression of specific genes was quantitated with the  $2^{-\Delta\Delta C_t}$  calculation according to the manufacturer's software (Bio-Rad; Livak and Schmittgen, 2001, where  $\Delta\Delta C_t$  is the difference in the threshold cycles of the specific gene and the reference housekeeping gene, which was ubiquitin (UBQ10) for expression analyses, and then the specific condition and the control condition to obtain relative transcript level. The mean threshold cycle values for the genes of interest were calculated from three technical replicates.

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## Supporting Information

**Table S1.** Calculation of the S3/S1 GFP ratio for Figure 1

	Relative GFP Transcript Level (log <sub>10</sub> )		S3/S1 ratio <sup>a</sup>	avgS3/avgS1 ratio <sup>b</sup>	<i>p</i> -value <sup>c</sup>
	S1	S3			
29B10	0.3 ± 0.2	1.1 ± 0.2	6.5 ± 0.6	6.4	1.1 e <sup>-08</sup>
29B25	2.34 ± 0.03	2.62 ± 0.03	7.4 ± 0.8	9.6	1.9 e <sup>-09</sup>
29B170	-1.5 ± 0.1	-0.6 ± 0.2	9.4 ± 2.3	4.9	1.5 e <sup>-06</sup>
29B171	-0.4 ± 0.2	0.4 ± 0.1	11 ± 1.6	13.4	9.1 e <sup>-10</sup>
29B172	2.6 ± 0.1	3.4 ± 0.2	2.1 ± 0.2	2.0	1.7 e <sup>-06</sup>

a. Calculated by the average of the S3/S1 ratio from individual plants, mean ± SEM.

b. Calculated by the ratio of the average of S3/average of S1.

c. Student's *t*-test for the difference between S1 and S3 was performed with the S1 and S3 log<sub>10</sub>-transformed values.

**Table S2.** Calculation of the S3/S1 GFP ratio for Figure 2

	Relative GFP Transcript Level (log <sub>10</sub> )		S3/S1 ratio <sup>a</sup>	avgS3/avgS1 ratio <sup>b</sup>	<i>p</i> -value <sup>c</sup>
	S1	S3			
ME1	3.36 ± 0.28	3.64 ± 0.31	18.86 ± 13.64	2.7	0.058
ME2	2.87 ± 0.24	3.03 ± 0.29	3.31 ± 1.33	2.1	0.045
ME3	2.27 ± 0.41	3.18 ± 0.46	64.03 ± 41.76	5.6	0.001
ME4	3.36 ± 0.36	4.23 ± 0.34	15.33 ± 5.43	5.7	0.000
ME5	5.21 ± 0.29	5.45 ± 0.36	8.72 ± 5.09	3.9	0.069
ME6	4.76 ± 0.28	5.25 ± 0.32	6.62 ± 2.78	3.7	0.001
ME7	3.73 ± 0.28	3.34 ± 0.34	3.01 ± 1.85	0.6	0.932
ME8	2.97 ± 0.27	2.18 ± 0.17	0.65 ± 0.28	0.0	0.153
ME10	3.25 ± 0.2	2.55 ± 0.28	0.38 ± 0.14	0.3	0.171
ME11	3.06 ± 0.25	4.14 ± 0.27	23.41 ± 10.19	14.7	0.000
ME12	2.34 ± 0.54	2.26 ± 0.45	1.74 ± 0.92	0.9	0.243
RD29A	0.62 ± 0.46	0.25 ± 0.41	1.11 ± 0.43	0.2	

a. Calculated by the average of the S3/S1 ratio from individual plants, mean ± SEM.

b. Calculated by the ratio of the average of S3/average of S1.

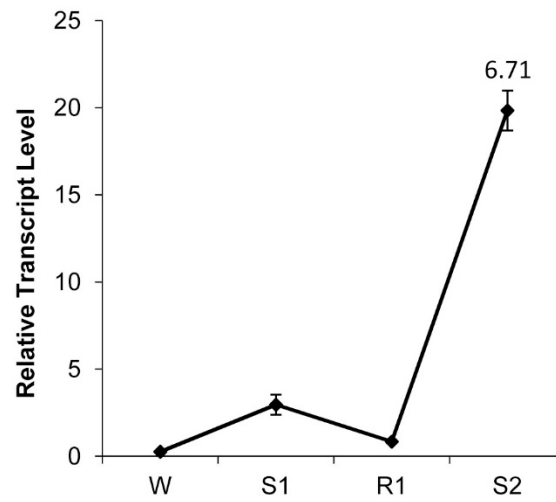
c. Student's *t*-test was performed with the ratio S3/S1 log<sub>10</sub>-transformed values against the RD29A controls for the independently transformed individual plants.

**Table S3.** See separate spreadsheet in Additional Files.

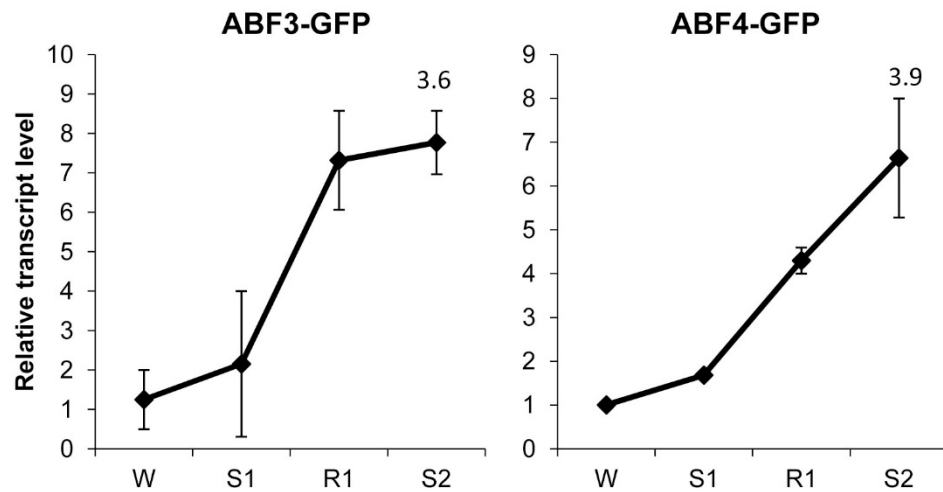
**Table S4.** Oligonucleotides used for the plasmid constructs

Gene Name/ Construction Name	Sequence (5' → 3')
ME1	GACCGtagccacgtagagagcaactggctgagacgtggcaggacgaaacggacgcCG GTCCGgcgccgttttcgtctgccacgtctcagccagttgtctctacgtggctaCG
ME2	GACCGgagagcaactggctgagacgtggcaggacgaaacggacgcatcgtacgtgtCG GTCCGacacgtacgatgcgtccgtttcgtctgccacgtctcagccagttgtctcCG
ME3	GACCGactggctgagacgtggcaggacgaaacggacgcatcgtacgtgtcagaatCG GTCCGattctgacacgtacgatgcgtccgtttcgtctgccacgtctcagccagtCG
ME4	GACCGacgtggcaggacgaaacggacgcatcgtacgtgtcagaatctacagaagCG GTCCGcttctgtaggattctgacacgtacgatgcgtccgtttcgtctgccacgtCG
ME5	GACCGacgaaacggacgcatcgtacgtgtcagaatctacagaagtaagagacaCG GTCCGtgtctctttactctgtaggattctgacacgtacgatgcgtccgtttcgtCG
ME6	GACCGgcatcgtacgtgtcagaatctacagaagtaagagacagaagccagagCG GTCCGctctggcttctgtctctttactctgtaggattctgacacgtacgatgcgCG
ME7	GACCGgtgtcagaatctacagaagtaagagacagaagccagagagaggtggtC GTCCGaaccacctctcttggtctgtctctttactctgtaggattctgacacCG
ME8	GACCGcctacagaagtaagagacagaagccagagagaggtggttcggccatatgCG GTCCGcatatggccgaaccacctctcttggtctgtctctttactctgtaggCG
ME10	GACCGactggctgaaatcaatcaggacgaaacggacgcatcgaatcaatagaatCG GTCCGattctattgattcgatgcgtccgtttcgtctgattgattcagccagtCG
ME11	GACCGgtaatcagtagctgtctgaagtagggtaagtatgctatacgtgtcgaggcCG GTCCGgcctcgacacgtatagcatacttaccctacttcagacacgtactgattacCG
ME12	GACCGagagttcgtacgtgtcagttatctggttcgcccacgtacgtgtctgcgaCG GTCCGtcgcagacacgtacgtcggcggaaccagataactgacacgtacgaactctCG
RD29A	GACCGaaatgactttgacgtcacaccacgaaaacagacgttcatacgtgtccctttatctcCG GTCCGgagataaaggacacgtatgaagcgtctgtttcgtgggtgacgtcaaagtcatttCG
5'end	tgggtgtatagtaagctcgtactgtagtagagcgacacgggtacgCG GTCCGcgtaccctgtctgcctctactacagtacgacttactataccacca
CaMV35S	GACCGgcaagaccttctctatataaggaagttcatttcatttggagagga tcctctccaaatgaaatgaacttcttatatagaggaagggtcttgcCG





**Figure S1.** Transcriptional memory responses of RD29B in an *abf2/3/4* triple mutant.



**Figure S2.** Analysis of the GFP-ABF3 and GFP-ABF4 transcript levels in response to successive dehydration stresses.

# **Method S1. RD29B Constructs 29B10, 29B25, 29B170, 29B171, and 29B172**

These constructs were made by standard recombinant DNA methods and have the following components and restriction sites:

**29B10:** 5'-BamHI/1.517 kb promoter of RD29B-5'UTR/XhoI/GFP/SacII/3'UTR of RD29B in a 1.4 kb 3' fragment of the 3' region of RD29B, followed by XmaI/KpnI sites.

**29B25:** 5'-BamHI/1.517 kb promoter of RD29B-5'UTR/XhoI/GFP/SacII/NOS3'/XmaI/KpnI.

**29B170:** The RD29B promoter region of 29B25 was PCR amplified in two fragments and combined by overlap PCR to contain a deletion between nucleotides -590/-352, and inserted as a 5'-BamHI/3'-XhoI fragment into the promoter region of 29B25.

**RD29B-590 primer:** 5'-CGATGCGAACTCGATTTCTCAACATCGat-3'

**RD29B-352 overlap primer:**

5'-CGATGTTGAGAAATCGAGTTCGCATCGGTTCAAAACAGCACACAGTTGA-TAGCTG-3'

**29B171:** A deletion between -590/-169 was created by PCR similarly to 29B170 and inserted as a 5'-BamHI/3'-XhoI fragment into the promoter region of 29B25.

**RD29B-169 primer:** 5'-CGATGTTGAGAAATCGAGTTCGCATCGTCGCATAGCCACGTAGAGAGCAACTG-3'

**29B172:** Synthetic oligonucleotides were used to create substitution mutations in the -169/+1 region of the RD29B promoter to give the following sequence: (substitutions in capital and TATA box underlined: RD2D B oligonucleotide: 5'-tcgcatagccacgtagagagcaactgGCTGAgacgtggcAGGACgaaacggacgcatcgtacgtgtcagaatcctacagaagtaaagagacagaagccagagagaggtgGTTTCGGCCATATGTCATCgttctctataaactttatggaactttgttctgattttctcagagacacgaaaagaaaga aaacaactagaaacaaagagggttgattgattca-3'/XhoI). This was combined by overlap PCR with the -1517 to -590 region of the RD29B promoter and inserted as a 5'-BamHI/3'-XhoI fragment into the promoter region of 29B25.

All of the constructs were excised as Bam/XmaI cassettes and inserted into a DsRED binary vector for *Agrobacterium*-mediated transformation of Arabidopsis. These binary plasmids were introduced into *Agrobacterium tumefaciens* by electroporation by using a GenePulser Xcell (Bio-rad Hercules, California, USA). Transformation of Arabidopsis plants was performed by a floral dip method (Bent 2006) and selected by red fluorescence on the seeds conferred by the binary vector (Stuitje et al. 2003). Four to six independently transformed plants were analyzed for each construct. The statistical analyses were performed by student's *t*-test on the log<sub>10</sub>-transformed values to compare the expression between the initial (S1) and the third (S3) stress (Table S1).